

Adherence of Dermatophyte Microconidia and Arthroconidia to Human Keratinocytes In Vitro

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The early interaction, adherence, between dermatophyte conidia and human keratinocytes has been studied in vitro. Two spore forms were used: microconidia and arthroconidia produced in vitro. The adherence of spores from three dermatophyte species, *Trichophyton rubrum*, *T. interdigitale*, and *T. quinckeanum*, was investigated using keratinocyte suspensions from different skin sites. Time-dependent adherence was demonstrated for all fungi studied with maximum adherence occurring between 3 and 4 h. There were no significant differences in adherence rates between the organisms studied. An order of affinity was established

between keratinocytes from different sites and significant differences were demonstrated in adherence of microconidia to skin cells derived from sole versus knee. No differences in adherence rates were demonstrated in atopics versus patients with chronic dermatophytosis and normals. Adherence was inhibited, but not abolished, by subinhibitory concentrations of ketoconazole, itraconazole, and griseofulvin. The interaction between microconidia, arthroconidia, and keratinocytes was verified with scanning and transmission electron microscopy. *J Invest Dermatol* 89: 529-534, 1987

Dermatophyte infections are caused by mold fungi of the genera *Trichophyton*, *Microsporum*, and *Epidermophyton*, which share the ability to invade stratum corneum and keratinized structures such as hair and nails derived from epidermis. In human infections, the organisms are derived from other human, animal, or soil sources. Infection is believed to follow indirect or direct contact with infected scales or hairs, and the chances of successful transmission are considerably improved by the prolonged viability of dermatophyte arthrospores in exfoliated skin [1]. Invasion of the stratum corneum is followed by proliferation of organisms with extension of the infection, in some instances, to other structures such as hair or nail [2]. The effectiveness of the resulting host-inflammatory response largely depends on active T cell mediated immunity or polymorphonuclear leukocytes depending on the site of infection [3]. Although various components of the host parasite relationship have been explored in detail, the initial phase of infection, the contact or adherence of fungi to host cells, has not been investigated.

Adherence of bacteria and *Candida albicans* to epithelial surfaces is thought to be a critical event in the development of infection

[4]. In the case of *C. albicans*, the process can be modified by changes in a large number of environmental conditions such as surface pH, presence of bacteria, and drugs [5]. Most studies have concentrated on the interrelationship between fungal and vaginal or buccal mucosal cells, although there has been one investigation of the adherence of candida cells to human stratum corneum [6]. The conditions necessary for the adherence of dermatophytes to human epithelial cells have not been investigated, however. For this reason, we have studied adherence of dermatophyte microconidia and arthroconidia to human stratum corneum to investigate the kinetics of attachment of the fungal cells and the effects of modifying local conditions on this process.

MATERIALS AND METHODS

Cultures Clinical isolates of *Trichophyton rubrum* (ID85/327) and *T. interdigitale* (ID85/149) were obtained from St. John's Hospital, London, U.K. for Diseases of the Skin, in addition to a reference strain of *T. quinckeanum* (NCPF 309) from the Mycological Reference Laboratory, Public Health Laboratory Service, Colindale, U.K. The correct designation of *T. quinckeanum* and *T. interdigitale* is still the subject of some disagreement [7]. For instance, both have been assigned to the imperfect species *T. mentagrophytes*. A control organism, *Aspergillus fumigatus* (NCPF 2210), was also used. All isolates were subcultured on malt agar at 30°C for 12 days prior to use.

Spore Suspensions

Microconidia: Between 5 and 8 ml of phosphate-buffered saline (PBS) (pH 7.3) was pipetted onto 12-day-old cultures of organisms grown on malt agar plates at 25°C. The surface of the colony was gently brushed with a glass rod. Spores were prepared by filtering the resulting suspension through an 8 µm diameter filter (Nuclepore Corp, Pleasanton, California) to remove larger fragments. Before each assay, fungal microconidia were washed in PBS and resuspended in TC medium 199 (Gibco Ltd, Livingstone, Scotland) to yield a suspension containing 1×10^7 spores/ml, the concentration of which was adjusted using a hemocytometer.

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Abbreviations:

CFUs: colony forming units
DMSO: dimethyl sulfoxide
MIC: minimum inhibitory concentration
PBS: phosphate-buffered saline
SEM: scanning electron microscope
TEM: transmission electron microscope
YNB: yeast nitrogen broth

The viability was checked by subculture on malt agar plates and colony forming units (CFUs) were counted. Conidia from *A. fumigatus* were prepared under similar conditions and used as control spores at the same concentration.

Arthroconidia: The production of arthroconidia from strains of *T. mentagrophytes* has been described previously [8]. The method used was based on a modification devised by Dr. Karl Clemons of the Division of Infectious Diseases, Santa Clara Valley Medical Center, San Jose, California. The culture used was a human isolate of *T. interdigitale* (ID 85/149). Isolates were grown on glucose peptone agar for 12 days, until a good yield of microconidia was available. The plate was flooded with PBS and the resulting suspension of microconidia plated evenly onto a glucose peptone agar plate and incubated at 37°C in a moist atmosphere within a sealed glass jar containing 5% carbon dioxide generated by a Gas kit (Oxoid Ltd, Basingstoke, U.K.). Plates were harvested at two weeks and the resulting growth removed by gently scraping the surface of the agar with a glass rod. The cells were resuspended in PBS, washed, and gently agitated. The suspension was allowed to settle for 30 min, the supernatant decanted, and filtered through a 12 μ m pore size Nuclepore filter. The effluent was centrifuged and resuspended in TC medium 199. The concentration of units (single arthrospores plus small arthrospore chains) was standardized at 1×10^7 units/ml, using a hemocytometer.

Preparation of Human Corneocytes: Keratinocytes were obtained from healthy nonatopic volunteers by the following methods. The skin was cleaned with 70% alcohol and lightly scraped with scalpel. The cells were suspended in PBS and vigorously shaken until a predominantly single cell suspension was obtained. They were checked microscopically and contained only nonnucleated

corneocytes. Cells were washed three times in PBS to remove adherent microorganisms, resuspended in TC medium 199 to give a final suspension of 1×10^6 cells/ml, and assessed in a hemocytometer. Suspensions were used within 2 h of preparation. Skin samples were taken for comparison from the dorsum and palm of the hand, forearm (ventral surface), knee, and sole of the foot.

In addition, skin samples were taken from eight patients with a personal or family history of atopy (hay fever, atopic eczema, or asthma) and eight patients with chronic dermatophytosis caused by *T. rubrum*, as well as nonatopic controls [6]. All scrapings were taken from the palms of uninfected hands using the method described previously.

Adherence Assay: The adherence assay used was a modification of a system described by Ray and co-workers [6]. Aliquots (0.2 ml) of a spore suspension containing 2×10^6 cells were mixed with a similar volume of the corneocyte suspension containing 2×10^5 cells and incubated at 37°C in a rotary shaker at 100 rpm for 4 h (New Brunswick Scientific, Edison, New Jersey). The cells were washed three times with PBS to remove excess non-adherent spores and passed through a 12.5 μ m diameter membrane filter (Millipore, Molsheim, France). The retained cells were resuspended in PBS, spun down, and placed on microscope slides. They were stained with Parker royal blue ink (1:10) and examined by light microscopy. The total number of conidia adhering to 100 cells was determined in triplicate per experiment and the mean number of spores attached to 100 cells calculated at 1, 2, 3, 4, and 6 h. Clumped or visibly damaged fungal cells were excluded from the count. Statistical analysis was carried out using Student's *t* test. For assays of atopic skin, only *T. interdigitale* spores were used.

A similar procedure was used for the arthrospore adherence

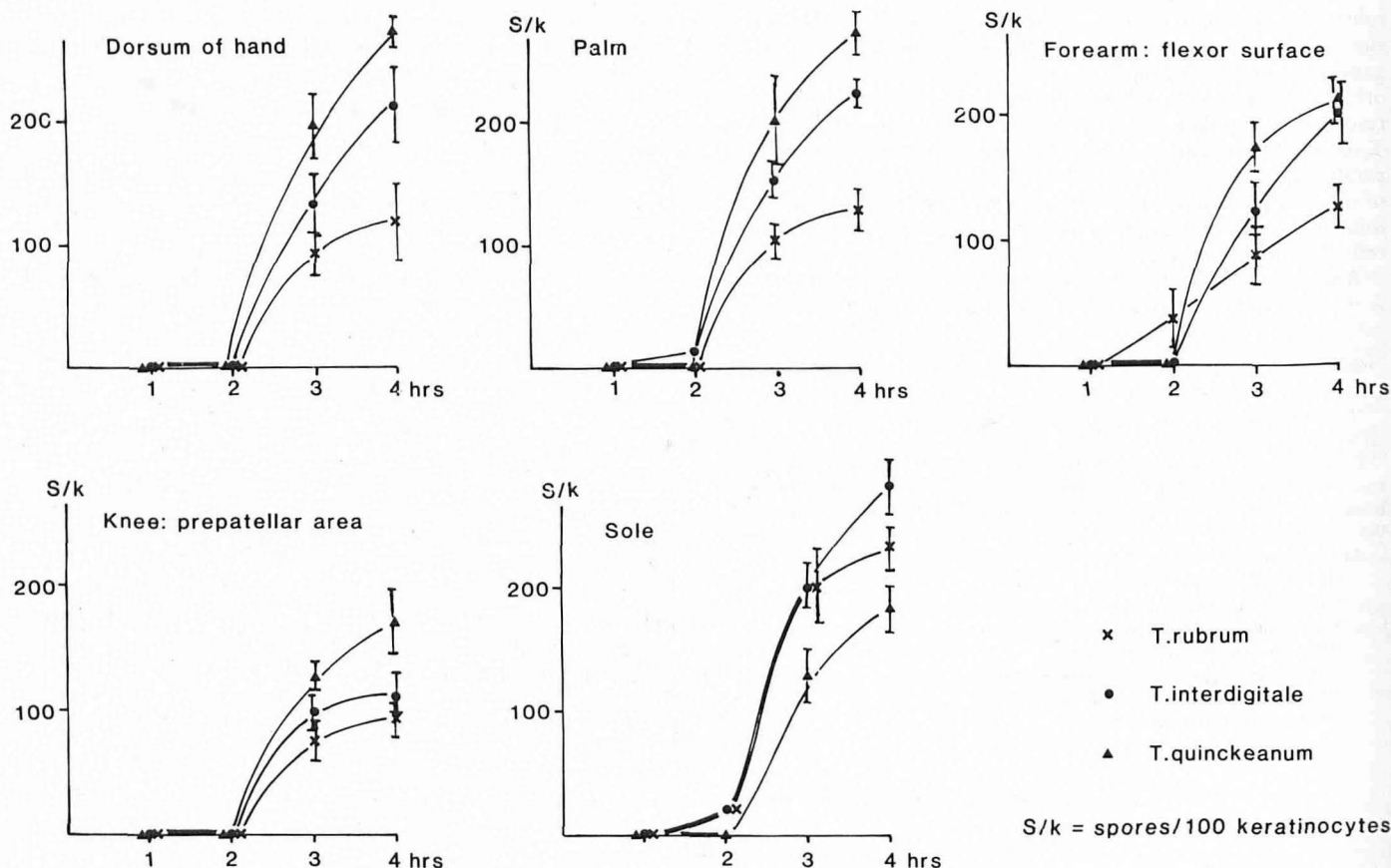


Figure 1. Time dependent adherence of microconidia from *T. rubrum*, *T. interdigitale*, and *T. quinckeanum* to keratinocytes derived from different skin sites. (Means and standard errors are shown).

assay. As it was not possible to separate some fungal cells using the methods described, however, the fungal suspension was standardized at 2×10^6 units. As individual units contained occasional three cell chains, accurate quantification of adherence was difficult, but samples were processed for transmission and scanning electron microscopy as described below. If two or more arthrospores were joined together, they were counted as one unit for the purpose of the assay.

Scanning and Transmission Electron Microscopy: Samples of adherence assays using both micro- and arthroconidia were processed for both scanning (SEM) and transmission (TEM) electron microscopy. For scanning electron microscopy, samples were filtered through a membrane filter ($0.5 \mu\text{m}$ polycarbonate, Millipore SA, Molsheim, France), washed three times with 0.075 M sodium cacodylate buffer ($\text{pH } 7.4$), fixed with 3% glutaraldehyde in the same buffer at 4°C for 1 h , and postfixed with 1% osmium tetroxide for 1.5 h before a final wash in cacodylate buffer. Fixed samples were dehydrated and coated before examination using a scanning electron microscope (JEOL 25 SEM).

Pellets of material were also fixed in glutaraldehyde as described above, embedded in 1% agar, washed, and postfixed in 1% osmium tetroxide before dehydration and staining with 1% uranyl acetate. Samples were embedded in epoxy resin. Sections on copper grids were stained with lead citrate before examination using a transmission electron microscope (AEI 801).

Drug Inhibition Assay: Three antifungal drugs were used. Ketoconazole (Janssen Pharmaceuticals, Wantage, U.K.) was prepared by dissolving 10 mg pure substance in 10 ml $1:1$ distilled water and acetone to give a stable solution containing $1000 \mu\text{g/ml}$ of drug. A similar procedure was used for griseofulvin (ICI, Macclesfield, U.K.). Itraconazole (Janssen Pharmaceuticals) was dissolved in dimethyl sulfoxide (DMSO) to give a final concentration of $0.5\% \text{ wt/vol}$.

Keratinocytes were prepared as described previously from the palms of healthy volunteers. Microconidia of *T. interdigitale* (85/149) were used for the assay. A range of drug concentrations was used in each series of tests. The inocula used were 0.5 ml containing 5×10^5 human cells and 5×10^6 microconidia, respectively. Drug solutions were added to the human cell suspension immediately prior to mixing. Control wells containing solvent (acetone, DMSO) without drug were included. Incubation was carried out at 37°C for 4 h . The adherence assay and counting procedure have been described above.

The minimum inhibitory concentrations (MICs) for each drug were determined for *T. interdigitale* (85/149) using a method described previously [9]. Inocula of microconidia were grown on yeast nitrogen broth (YNB) in microtiter plates and endpoints were defined visually.

RESULTS

Adherence of Microconidia to Human Keratinocytes These studies show that microconidia of all three dermatophytes adhere to human keratinocytes in a time-dependent fashion (Fig 1). The adherence occurs after $2\text{--}3 \text{ h}$ of incubation and further significant increases do not occur after 4 h , although after this period germination of spores takes place, making an accurate assessment difficult. In most assay systems, *T. quinckeanum* showed the best adherence except in tests involving keratinocytes derived from soles where *T. interdigitale*, followed by *T. rubrum*, showed greater adherence. These differences, however, were not statistically significant.

The figures show that there were also quantitative differences in adherence of microconidia to keratinocytes derived from different sites (Fig 1). At 3 h adherence of spores from all three isolates used in descending order was as follows: sole > palm > dorsum of hand > forearm > knee. It was only possible, however, to show significant differences for *T. rubrum* and *T. interdigitale*,

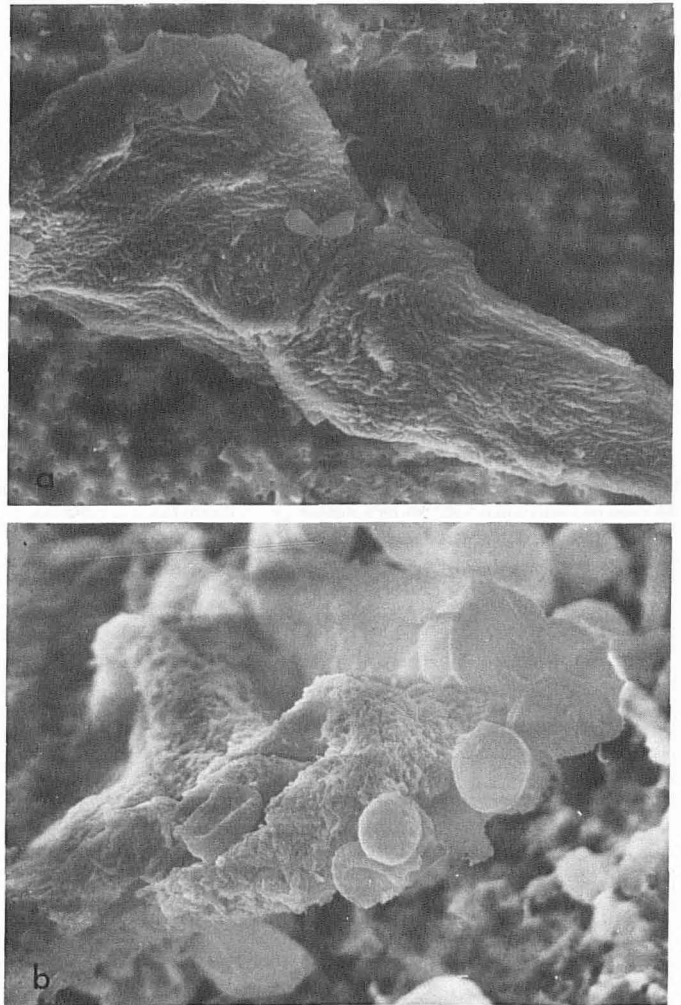


Figure 2. a, SEM of small microconidia of *T. interdigitale* adhering to human keratinocytes. ($\times 1500$). b, SEM of arthroconidia of *T. interdigitale* clustered around the edge of a keratinocyte. ($\times 1500$).

but not *T. quinckeanum*, when adherence of conidia to keratinocytes from soles was compared with that from knees ($p < 0.005$).

Conidia from *A. fumigatus* showed low adherence rates of $22 (\pm 5)$ and $40 (\pm 7)$ spores per 100 keratinocytes for cells derived from soles at 3 and 4 h , respectively. Fewer than 10 cells were adherent per 100 keratinocytes before 2 h .

Adherence of Arthroconidia to Human Keratinocytes Adherence of arthroconidia of *T. interdigitale* to keratinocytes from palmar or forearm skin showed a time dependent relationship with maximum adherence occurring by 3 and 4 h (128 ± 18 and 152 ± 21 units per 100 forearm cells and 95 ± 8 and 120 ± 23 per 100 palmar cells, respectively). The pathological significance of these findings is unclear, however. Some smaller cells are retained which show the morphologic features of microconidia and it is not known whether this will affect their adherence properties.

Dermatophyte Adherence in Atopic Patients and Those With Chronic Dermatitis The mean adherence of *T. interdigitale* microconidia to palmar skin from atopic patients was 187 ± 48 in five replicate experiments (spores/100 keratinocytes). Those with chronic dermatophytosis showed a mean adherence of 159 ± 25 . These values do not differ significantly from those obtained in control studies (215 ± 51).

Ultrastructural Studies of Conidial Adherence Microconidia could be seen to adhere closely to the surface of keratinocytes using SEM (Fig 2a). Sometimes clusters of microconidia could be seen. With SEM the rough outer layer of the keratinocytes was demonstrated (Fig 2a). Using transmission electron microscopy the surface of microconidia appeared to be closely opposed to underlying human cells (Fig 3). The outer layer of the microconidial cell wall was seen to consist of concentric electron dense zones alternating with electron lucent zones. This outer rim had a scalloped appearance. Close apposition to fibrillar material in the underlying keratinocyte could be seen, and in places it was not possible to define a border between the two, suggesting true physical adherence.

With arthroconidia, cell shapes varied (Fig 2b) although the majority were of uniform size and some short chains of cells were shown to adhere to keratinocytes. At 4 h, some germination had occurred, which suggested that hyphal formation in situ may develop more rapidly from arthrospores compared with microconidia. In transmission electron microscopic studies, the characteristic walls of arthrospores could be demonstrated, and their appearances were compared with those of the microconidia (Fig 4). The main difference observed was the thickness of the arthrospore cell wall. It did not show projections or similar features that might affect adherence.

Effect of Antifungal Drugs on Dermatophyte Adherence

The MICs of ketoconazole, itraconazole, and griseofulvin for *T interdigitale* were found to be 1.0, 0.6, and 0.3 $\mu\text{g}/\text{ml}$, respectively. It was found that the inhibition of adherence was dependent on the concentration of drug but that significant inhibition occurred when the concentrations of compounds were as low as 0.01 $\mu\text{g}/\text{ml}$, a value that was found to be considerably lower than the normal MIC range for the drugs and organisms concerned. Only at the

lowest concentration of griseofulvin, 0.01 $\mu\text{g}/\text{ml}$, was there no significant difference between test and control assays.

DISCUSSION

We have found that microconidia derived from three human dermatophyte species, *T rubrum*, *T interdigitale*, and *T quinckeanum* showed time-dependent adherence to human keratinocytes in vitro. There were differences in adherence rates between the different organisms using human cells from each site tested, but these did not achieve statistical significance. Likewise, a decreasing frequency of adherence of fungal conidia to skin cells from soles, palms, dorsum of hand, forearm, or knee was shown. The difference was statistically significant only when the two most disparate assays, involving sole and knee keratinocytes, were compared for *T rubrum* and *T interdigitale*. Although inocula of microconidia are relatively easy to standardize, they are not the most appropriate fungal propagules for testing adherence, as the form implicated in naturally acquired infections is presumed to be the arthrospore. A method of producing arthrospores in an atmosphere containing 5–8% carbon dioxide has been described previously [8], and a modification of this technique was used to study adherence that appeared to be similar to that seen with microconidia. Arthrospores differ from the microconidia in shape, size, and in thickness of the cell wall (Fig 4). In addition, small chains of arthroconidia may form. These differences did not appear to lead to more rapid adherence to the corneocyte substrate, however.

Reports of adherence of fungi to mucosal surfaces or skin are few and most studies have involved *Candida albicans* [4–6]. Although yeast cells of the latter organism show adherence, the formation of germ tubes is also important in promoting surface

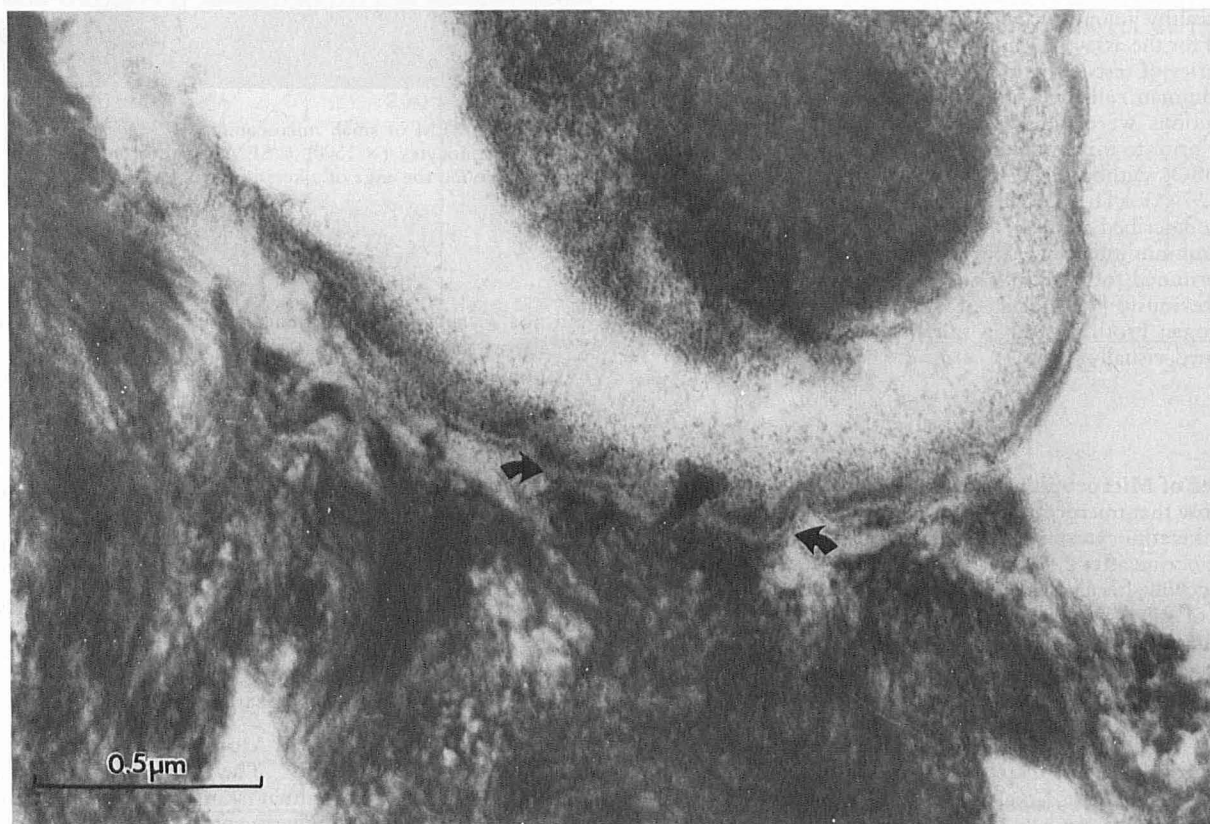


Figure 3. Transmission electron microscopy of *T interdigitale* microconidia adhering to a human keratinocyte. There is close apposition between the outer electron dense zone of the fungal cell wall and the keratin matrix of the human cell (arrows). ($\times 60,000$).

adhesion [4]. To our knowledge, there are no comparable studies for dermatophytes. Significant adherence between *C. albicans* blastospores and mucosal cells usually occurs within 1 h [4,5], considerably earlier than seen in the present study with dermatophytes. The latter results may have been affected by two factors. First, adherence of fungi to corneocytes may proceed at a slower rate. In one previous investigation of adherence of *Candida* species to keratinocytes [6], significant adherence was generally delayed until after 1 h. In addition, dermatophyte conidia in common with species of other fungi, such as zygomycetes [10], undergo a swelling reaction prior to germination, which may well affect the adherence properties. After 2 h of incubation in TC medium, *T. quinckeanum* microconidia show a mean increase in length of 19% (± 8) measured by a microscope eyepiece micrometer graticule. These two factors may well account for the slow onset of adherence. Germination of spores is not a critical factor in this process, however, as this occurs after 4 h of incubation. A number of investigators have shown that adherence of *Candida* is reduced by the presence of lactobacilli [4] and other bacteria. By contrast piliated strains of *Klebsiella pneumoniae* have been shown to enhance adherence of *Candida* to epithelial cells [1]. The normal epidermis supports a large microflora mainly consisting of coryneforms, *Staphylococcus albus*, and *Propionibacterium acnes*. It is likely that these may also interact during the adherence process. We used alcohol swabbed skin, which may be sufficient to remove some superficial bacteria; this was confirmed by microscopy, but the effects of the normal flora on the adherence process need to be studied in more detail. Other factors that affect adherence of *C. albicans* to the underlying substrate include strain [12], ambient pH [13], and state of germination [4].

In human dermatophyte infections, certain patients, particularly atotics, appear to be more susceptible to chronic disease. We have not been able to detect any increase of adherence of dermatophytes to skin from atopic subjects or those with chronic dermatophytosis compared with normal controls to account for increased susceptibility. Interestingly, enhanced adherence of *Staphylococcus aureus* to atopic cells has been cited as a possible explanation for the high carriage rate of this bacterium in atopic eczema. Likewise, cells from individuals with chronic dermatophyte infections did not interact more readily with fungi. In view of the poor immune response seen in these patients, it is more likely that factors occurring after invasion, such as T cell reactivity, are more important in determining susceptibility [3]. This view is supported by the observation that in a group of individuals with a high transmission rate for dermatophytosis, coal miners, there was no increased prevalence in atopy in infected versus noninfected subjects [14]. Alternatively, other variables, such as sebum or sweat composition, may affect invasion.

With *C. albicans* it has been shown that pretreatment of cells with antifungal agents decreases adherence if the organisms are in mycelial phase [15] but not in stationary yeast phase. We have found a similar phenomenon using ketoconazole, itraconazole, and griseofulvin with dermatophyte microconidia. Although it may be supposed that decreased adherence is a consequence of reduced viability in *C. albicans*, adherence can be seen with killed *Candida* cells, although it is variable [16]. In our system, adherence occurs at drug concentrations considerably below the normal range of MICs for each drug, but is less than that seen in the absence of drug. This suggests that surface changes presumably affecting the cell wall are important for adherence, that they may occur at subinhibitory concentrations of the drug, and that these do not necessarily reflect the prime site of activity of the compounds in prevention of cell growth. In the case of the azole drugs, this is the inhibition of cytochrome P450 dependent cell membrane synthesis, and with griseofulvin, the inhibition of intracellular microtubule formation. Inhibition of filamentation may be important at a later stage but does not explain the loss of adherence shown here, which occurred before filaments develop.

Finally, the contact between dermatophyte spores and keratinocytes depends on close apposition of cells. Electron micros-

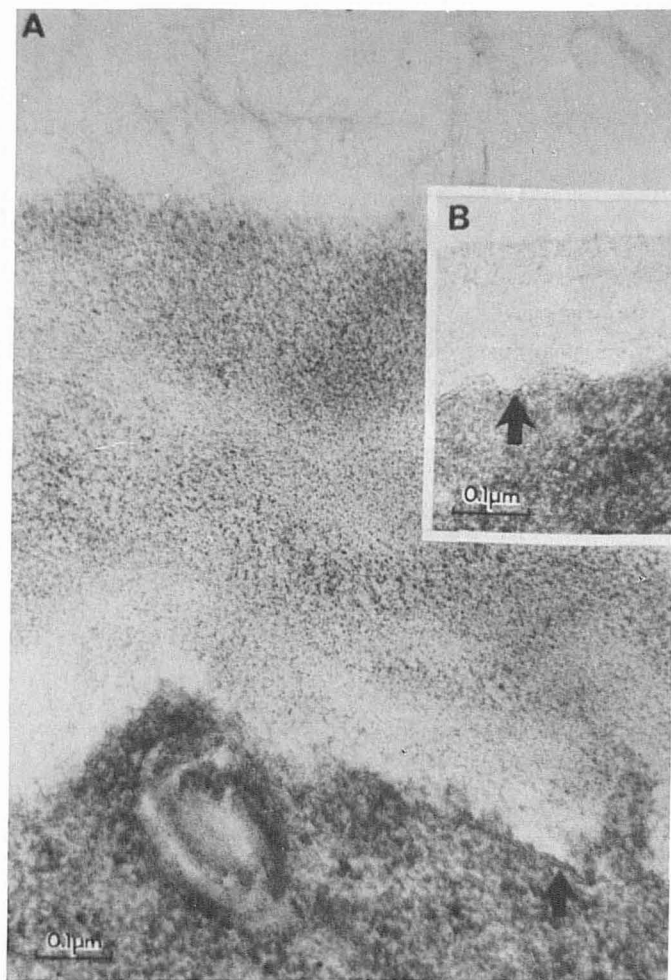


Figure 4. A comparison of the transverse sections of microconidial (B) and arthroconidial (A) cell walls at the same magnification under TEM. ($\times 120,000$). The cell membrane is arrowed.

copy shows that it is not possible to clearly define the border between the cells and it appears to be filled with amorphous electron dense material. With *C. albicans*, adherence depends on the presence of specific adhesins, which are thought to be glycoproteins [17], the effect of which can be blocked by certain substances, such as simple sugars (e.g., fucose) but enhanced by others (e.g., glucose, galactose) [18]. Sucrose mouth washes can aggravate candida stomatitis possibly through a similar mechanism [19]. The pathologic significance of these observations is not clear, however, as at high concentrations the inclusion of these sugars in the yeast growth medium may stimulate adherence in vitro. On the skin surface there are other factors such as the medium chain length fatty acids derived from sebum. Their presence is important in regulating the relative concentrations of different bacteria and they are believed to play a role in inhibiting the growth of dermatophytes accounting, for instance, for the rarity of scalp ringworm after puberty [20]. The effect of fatty acids on dermatophyte adherence may be an important regulatory factor that could account, for instance, for the difference in adherence of dermatophyte conidia to keratinocytes from sites such as the soles of feet compared with the knees. The latter area contains sebaceous glands and sebum, which is likely to be retained on the surface of keratinocytes, whereas the former site is devoid of sebaceous glands. This question is presently under study.

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